Studies on synaptosomal adenylate cyclase and its stimulation by the supernatant from rat cerebral cortex(ラット大脳皮質におけるシナプトソームadenylate cyclaseと、その上清による活性化に関する研究)

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Studies on Synaptosomal Adenylate Cyclase and its Stimulation by the Supernatant from Rat Cerebral Cortex.

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CHAPTER 1 INTRODUCTION

Adenosine 3', 5'-monophosphate (cyclic AMP) was discovered by Rall and Sutherland in the course of their investigation on the role of epinephrine and glucagon in the breakdown of glycogen in liver (Rall and Sutherland 1957; Rall and Suterland 1958; Sutherland and Rall 1958). The substrate adenosine triphosphate (ATP) in the presence of Mg^{++} is enzymatically cleaved to form cyclic AMP and pyrophosphate (Rall and Sutherland 1962) (Fig. 1-1). The enzyme catalyzing this reaction, adenylate cyclase (Sutherland et al. 1962), was found in the plasma membrane of almost all species of animals (Sutherland et al. 1962). A phosphodiesterase which is found in both the soluble and particulate fractions of the cell, hydrolyzes cyclic AMP to 5'-monophosphate (Sutherland and Rall 1958).

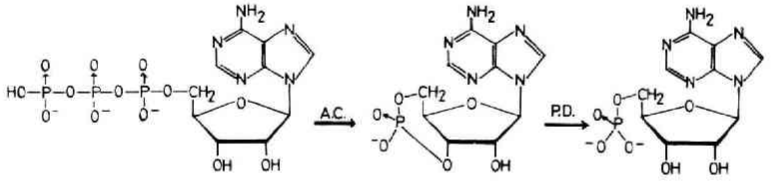


Fig. 1-1. The reactions catalyzed by adenylate cyclase and cyclic AMP phosphodiesterase. A.C: Adenylate cyclase. P.D: Cyclic AMP phosphodiesterase.

Cyclic AMP is widely distributed in nature and has been detected in several bacteria (Hirata and Hayaishi 1965; Makmann and Sutherland 1965; Brana 1969; Tao and Lipmann 1969), slice mold (Konijin et al. 1967), higher plants (Pollard 1970; Duffus and Duffus 1969; Azhar and Hrishnamurti 1971), and almost all animal tissues so far studied (Ebadi et al. 1971). Interaction of a number of polypeptide hormones and biogenic amines with their respective target tissues leads to an enhanced production of cyclic AMP in these cells. The increase in the level of cyclic AMP precedes to all metabolic and physiological response elicited by hormones. These observations led Sutherland et al. to propose cyclic AMP as a second messenger in hormone action (Sutherland et al. 1965; Robison et al. 1967; Sutherland et al. 1968). Cyclic AMP alters metabolic and physiological responses either directly or indirectly by releasing certain hormones (Rasmussen and Tenenhouse 1968) which in turn may act via cyclic AMP on their target tissues.

Various hormones are known to be capable of stimulating adenylate cyclase. It has been reported that the following criteria shoud be satisfied in order to justify a claim that a given hormone produce a given effect as a result of this action (Robison et al. 1971).

(1) The hormone should be capable of stimulating adenylate cyclase in broken cell preparations from the approciate cells, while hormones which do not produce the response should not stimulate adenylate cyclase.

(2) The hormone should be capable of increasing the intracellular level of cyclic AMP in target cells, while inactive hormones should not increase cyclic AMP levels. It should be demonstrated that the effect on the level of cyclic AMP occurs at dose levels of the hormone which are at lease as small as the smallest levels which are capable of producing a physiological response. The increase in the level of cyclic AMP should precede or at least not follow the physiological response.

(3) It should be possible to potentiate the hormone (i.e., increase the magnitude of the physiological response) by administering the hormone together with theophylline or other phosphodiesterase inhibitors. The hormone and the phosphodiesterase inhibitor should act synergistically.

(4) It should be possible to mimic the physiological effect of the hormone by the addition of exogenous cyclic AMP.

In brain, the amines such as epinephrine, norepinephrine, histamine, and serotonin stimulate cyclic AMP formation (Sattin and Rall 1967, 1970 ; Kakiuchi and Rall 1968a, b ; Schimizu et al. 1969, 1970a, b, c). Information regarding the hormonal regulation of cyclic AMP formation in brain is lacking due to two difficulties : (a) within a few seconds after death the level of cyclic AMP in brain increases several-fold, unless special care is taken to freeze the tissue quickly (Kakiuchi and Rall 1968a ; Paul et al. 1970). (b) The enzyme in broken cell preparations loses its response to activation by hormones. Decrease in hormone response in broken cell preparations is observed in some tissues (Forn et al. 1970) but not in others (Moskowitz et al. 1970). For these reasons, brain slices have been used for studies of cerebral adenylate cyclase in vitro. In such experiments, slices are preincubated with ^{14}C- or ^{3}H-adenine, and the formation of cyclic AMP is monitored from the prelabeled pool of ATP. During typical experiments, in which rat cerebral cortex slices preincubated with adenine are incubated in the presence of norepinephrine, 5-6% of labeled ATP is converted to cyclic AMP in the first minutes. In terms of percentage of total ATP converted to cyclic AMP, the conversion is about 0.5%. These observations suggest that there are several pools of ATP in slices, and at least one pool is preferentially labeled when incubated with adenine. Possibly ATP in this pool is easily accessible to adenylate cyclase, which is stimulated by norepinephrine (Krishna et al. 1970).

As mentioned above, in contrast to most tissues, broken cell preparations of adenylate cyclase from brain tissue do not respond to neurohormones with increase in the rate of synthesis of cyclic AMP (William et al. 1969 ; Rall and Sattin 1970 ; McCune et al. 1971). However, brain has the highest capacity for synthesizing cyclic AMP (Sutherland et al. 1962) as well as the highest levels of cyclic nucleotide phosphodiesterase (Butcher and Sutherland 1962). For these reasons, many workers have investigated the role of cyclic AMP in the central nervous system.

In light of observations (Bitensky et al. 1968 ; Oye and Sutherland 1966) that similar loss of hormone sensitivity was seen in other tissue homogenates, it seems likely that some essential substances for the stimulation of the enzyme by such neu-

rotransmitters are removed from the membrane during homogenization and fractionation of the tissues. There is little data, however, on these substances and whether or not they have any influence on the adenylate cyclase. In the experiments published so far the particulate fraction was suspended in buffer solution without the presence of soluble supernatant fraction.

In the present thesis, the effect of the $105{,}000 \times g$ supernatant fraction (supernatant) and the effects of combination of the supernatant and various agents such as neurotransmitters and sodium fluoride, which is the well-known potent activator of adenylate cyclase, on the adenylate cyclase of rat cerebral cortex were investigated (Izumi and Ozawa 1975; Izumi et al. 1975a; Izumi and Ozawa 1976; Izumi et al. 1976e).

Chapter 2. Assay of Adenylate Cyclase

Early work on adenylase cyclase relied on biological assays of cyclic AMP (Rall and Sutherland 1958, 1962). These techniques were tedious and usually involved extensive purification of cyclic AMP samples since they involved multicomponent enzyme system whose activity could be influenced by a number of adenine nucleotides and certain hexoses. With the availability of radioactively labeled ATP and the advent of quick, simple methods for the purification of cyclic AMP, investigators were provided with a direct radiometric assay procedure for adenylate cyclase activity in broken cell preparations (Rabinowitz et al. 1965; Streeto and Reddy 1967; Krishna et al. 1968; Barr and Hechter 1969).

The most widely used method for the isolation of cyclic AMP from adenylate cyclase reaction mixtures is that of Krishna et al. (1968). The procedure involves chromatography of the samples on a column of Dowex 50 followed by the precipitation of containing nucleotides with $ZnSO_4$ and $Ba(OH)_2$.

In the present experiments, a modified method of Krishna et al. (1968) was utilized and ^{3}H-ATP was used as a substrate of the reaction system in the assay of adenylate cyclase activity. In case of low purity of a commercial ^{3}H-ATP, it is sometimes necessary to purify it by column chromatography on Dowex 50W-H^+, or by a thin layer chromatography on silica gel. Then, the labeled ATP was mixed with unlabeled material prior to the assay to give the required specific activity.

The incubation medium for the assay of adenylate cyclase activity contains: Tris-HCl (tris (hydroxymethyl)aminoethane HCl) buffer, pH 7.4 (4×10^{-2}M); $MgSO_4$ (3.3×10^{-3}M); NaF (10^{-2}M); caffeine (6×10^{-3}M); ATP (10^{-3}M, 10 μCi) and enzymes (0.1–1.0 mg of tissue) in final volume of 0.6 ml. Test tubes (12×105 mm) containing all the ingredients for the assay of adenylate cyclase activity were preincubated for 5 min at 30°C prior to addition of substrate (ATP) which started the reaction. Incubations were carried out for 15 min at 30°C and terminated by the immersion of the test tubes in a boiling water bath for 2 min. As illustrated in Table 2-1, after addition of 0.1 ml of ^{14}C-cyclic AMP (about 5,000 dpm) as an internal standard for calculation of con-

Table 2-1.

Purification of Cyclic AMP

Incubation of the reaction medium at 30°C for 15 min

↓

Boiling for 2 min. then cooling

↓

Addition of internal standard
(^{14}C-Cyclic AMP, 5,000 dpm)

↓

Removal of ATP by the addition of $ZnSO_4$ & $Ba(OH)_2$
(twice)

Centrifugation (1,000 xg for 20 min)

PPT (Discard)

↓

Ion exchange column chromatography with
Dowex 50W X-4 (0.3 × 8 cm)

Fractionation (H_2O)

↓

Liquid scintillation spectrometory

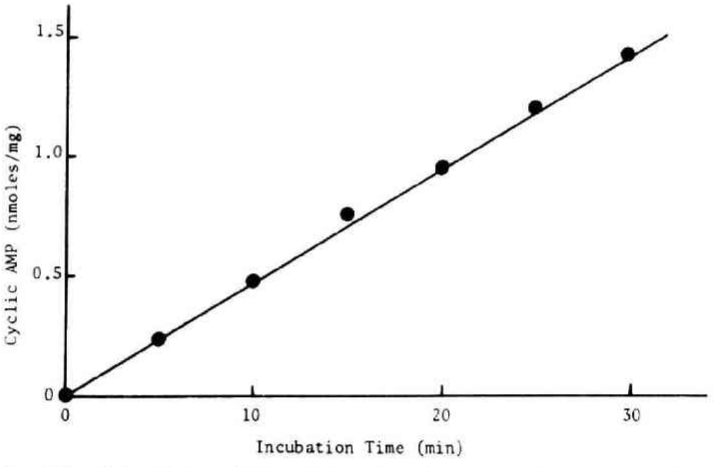


Fig. 2-1. Adenylate cyclase activity of rat brain synaptosomes as a function of time. Each sample contained synaptosomes (0.2 mg of protein) as enzyme sources. Incubation was conducted for various times at 30°C.

version of ^{3}H-ATP to ^{3}H-cyclic AMP, 0.1 ml of each of $ZnSO_4$ (0.17 M) and $Ba(OH)_2$ (0.15 M) solutions was added to each tube. The supernatant solution was transferred to another tube by decantation, and then the Ba-Zn precipitation was performed for a second time. The mixture was agitated and centrifuged at 1,000 × g for 10 min. The resulting supernatant was applied to a column of Dowex 50W-X4 in the hydrogen from (0.3 × 8 cm) and then cyclic AMP was eluted with water. Every 1 ml of the eluate was collected and transferred to a counting vial with 10 ml of scintillating fluid

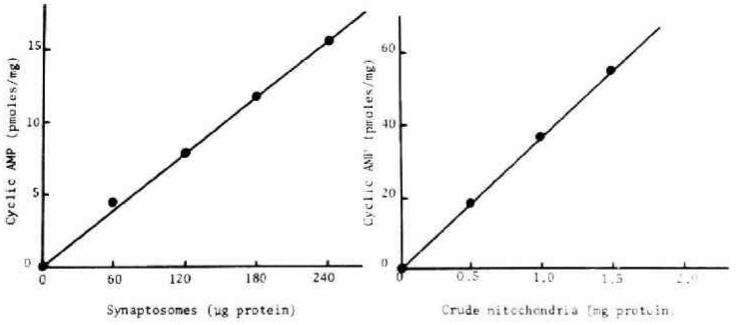


Fig. 2-2. Adenylate cyclase activity of synaptosomal and crude mitochondrial fractions from rat brain as a function of enzyme concentration.

which contained 4 g PPO, 100 mg dimethyl POPOP and 500 g triton X-100 in 1 liter of toluene. The radioactivity was determined by a liquid scintillation spectrometer (Packard-Carb Model 3380) and corrected for quenching using an external standardization system. The recovery of cyclic AMP was 75-80%.

Figure 2-1 shows the assay of adenylate cyclase of rat brain synaptosomes using ^{3}H-ATP as substrate as a function of time. The rate of formation of cyclic AMP was linear with time up to 30 min. Figure 2-2 shows the effects of varying protein concentrations upon the rate of cyclic AMP formation. In both enzyme preprations, the rate of cyclic AMP acumulation was proportional to protein concentration used.

Based on these experimental data, most experiments were carried out with a 15 min incubation time and with a protein concentration of 200 μg in 0.6 ml of incubation medium.

Chapter 3. Effects of the Supernatant on the Adenylatn Cyclase of Rat Cerebral Cortex

3-1. *Effect of Supernatant on Subcellular Fractions*

The effects of the supernatant on adenylate cyclase of subcellular fractions of rat cerebral cortex were investigated. The data in Table 3-1 show that the enzyme activity in crude mitochondrial fraction was significantly stimulated by the addition of the supernatant. Since the crude mitochondrial fraction contains the free mitochondria and a variety of isolated nerve endings (Whittaker 1959), this fraction was further fractionated into three fractions, i.e., myeline, synaptosomes and mitochondria by a sucrose density gradient ultracentrifugation methed (Whittaker 1959) in order to obtain more information about the effect of the supernatant on adenylate cyclase, and each enzyme activity was then determined in the absence and in the presence of the supernatant. As shown in Table 3-2, three particulate fractions showed similar enzyme activities in the absence of the supernatant, while upon addition of the supernatant the enzyme activity in synaptosmes was activated more markedly than those in

Table 3-1. Effect of supernatant on adenylate cyclase activity in subcellular fractions from rat cerebral cortex

Fractions	Adenylate Cyclase Activity (pmoles cyclic AMP/min per mg protein)	
	Without supernatant	With supernatant
1,000 xg supernatant (Whole homogenate)	71.7 ± 1.3	
10,000 xg sediment (Crude mitochondria)	61.8 ± 4.0	150.0 ± 4.6
105,000 xg sediment (Microsome)	92.0 ± 22.4	102.0 ± 5.1
105,000 xg supernatant	0	0

The subcellular fraction of rat cerebral cortex was obtained as described in Methods. The supernatant obtained after centrifugation at 105,000 xg, equivalent to 180 μg of protein, was added to each of the other fractions. Each value was the mean of 3 determinations of one fractionation.

Table 3-2. Effect of supernatant on adenylate cyclase activity in submitochondrial fractions

Fractions	Adenylate cyclase activity (pmoles cyclic AMP/min per mg protein)	
	Without supernatant	With supernatant
Mitochondria	21.3 ± 3.4	49.3 ± 2.8
Synaptosomes	38.8 ± 5.6	117.7 ± 1.3
Myelin	34.5 ± 12.1	63.1 ± 10.3

Fractions from rat homogenates (1,000 xg − 10,000 xg) was subfractionated by a discontinuous sucrose gradient centrifugation. The supernatant obtained as described in Methods, equivalent to 180 μg of protein, was added to each incubation medium. Each value was the mean of 3 determination of one fractionation.

the other two fractions. Fig. 3-1 shows that the adenylate cyclase activity in the crude mitochondrial fraction resides largely in the synaptosomal fraction (about 61%).

In order to determine whether or not the synaptosomal fraction described above is rich in nerve ending and other synaptic components, the morphologic study was undertaken. Electron micrograph (Fig. 3-2) of the synaptosomal fraction shows that there are numenous nerve endings and tiny axon terminals filled with typical synaptic vesicles. This fraction contains very little of myeline and mitochondria.

3-2. *Discussion*

It is well established that adenylate cyclase is stimulated by various neurotransmitters such as norepinephrine, 5-hydroxytryptamine (serotonin) and histamine in brain slice (Sattin and Rall 1967, 1970; Kakiuchi and Rall 1968a, b; Schimizu et al. 1969, 1970a, b, c; Chasin et al 1971a, b, 1973) and by direct administration of mono-

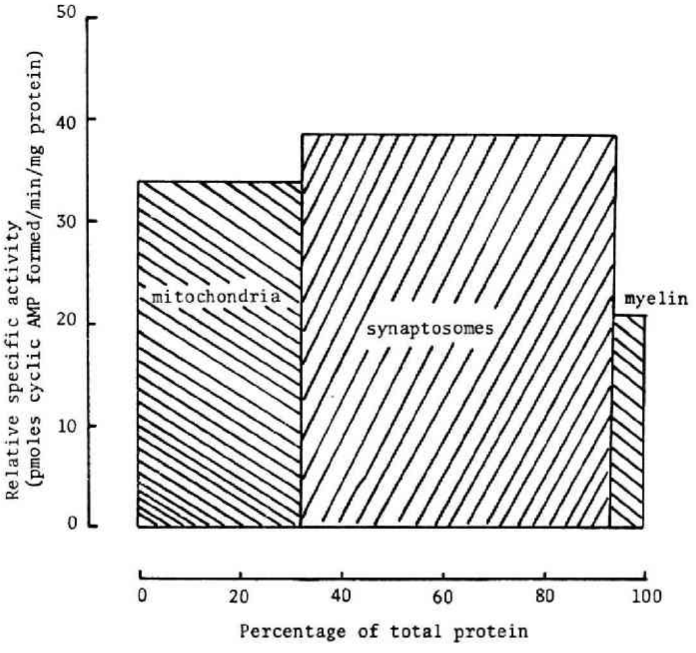


Fig. 3-1. Distribution patterns of adenylate cyclase in crude mitochondrial fraction. Ordinate: mean relative specific activity of fractions. Abscissa: fractions are represented by their relative protein contents.

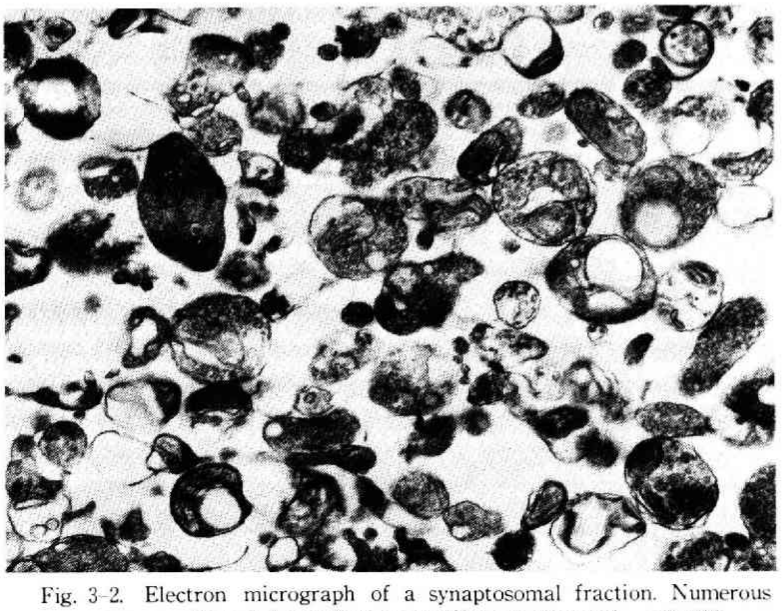


Fig. 3-2. Electron micrograph of a synaptosomal fraction. Numerous nerve endings and membranous profiles are observed. ×25,000.

amine into the central system (Chou et al. 1971). However, failure to obtain stimulation of brain adenylate cyclase by hormone and putative neurotransmitters in cell-free system has been reported (William et al. 1969 ; Rall and Sattin 1970 ; McCune et al. 1971). These data suggest that there are some other compounds, as yet unidentified, which may be the physiological stimulants for brain adenylate cyclase.

Previously, in the process of studying the distributions of adeylate cyclase of both bovine pineal gland and rat cerebellum, Weiss and Costa (1968) have found that in contrast to bovine pineal gland, fractionation of the cerebellum decreases the specific activity of the enzyme. Therefore, they have investigated the effect of the soluble supernatant material on the adenylate cyclase activity of subcellular fractions of rat cerebellar homogenates. It was found that the soluble supernatant materials, which itself had no adenylate cyclase activity, significantly enhanced the enzyme activity of the $11,500 \times g$ (crude mitochondrial) fraction but not that of other fractions. From these results, they suggested that the decrease in specific activity of subcellular fractions of cerebellar homogenates might be ascribable to a removal of some essential requirement or activator of the enzyme. Accordingly, the effectts of the $105,000 \times g$ supernatant (supernatant) on the adenylate cyclase activity of subcellular fractions of rat brain cerebral corebral cortex were investigated in order to gain more information about the involvement of the stimulatory substance(s) in the supernatant in other tissues such as rat cerebral cortex. As seen in Table 3-1, the enzyme activity of the crude mitochondrial fraction was markedly stimulated by the addition of the supernatant, which is in good agreement with those previously reported by Weiss and Costa (1968) concerning the supernatant stimulation of adenylate cyclase of mitochondrial fraction from rat cerebellum mentioned above. In light of the observations in Tables 3-2 and Fig. 3-1, this increase of cyclic AMP accumulation may be due to largely to the stimulation of the synaptosomal adenylate cyclase by the supernatant. These findings suggest that adenylate cyclase of synaptosomes may be different from the enzyme present in other particulate fractions of rat cerebral cortex, and that the adenylate cyclase of synaptosomal membranes may be regulated by endogenous substances in the rat brain. For these reasons, the properties of synaptosomal adenylate cyclase and the characterization of the stimulatory substance(s) in the supernatant were investigated, and the results are described in the following chapter.

Chapter 4. Characterization of Stimulatory Substance (*s*) in the Supernatant

4-1. *Effect of Concentration of the Supernatant*

The relationship between the degree of the stimulation and the concentration of the supernatant is depicted in Fig. 4-1. As the concentration of the supernatant increased, the accumulation of cyclic AMP progressively increased, reaching a plateau at a concentration of about 200 μg protein. In the assay of the stimulatory activity described in the present studies, preliminary experiments were usually carried out to

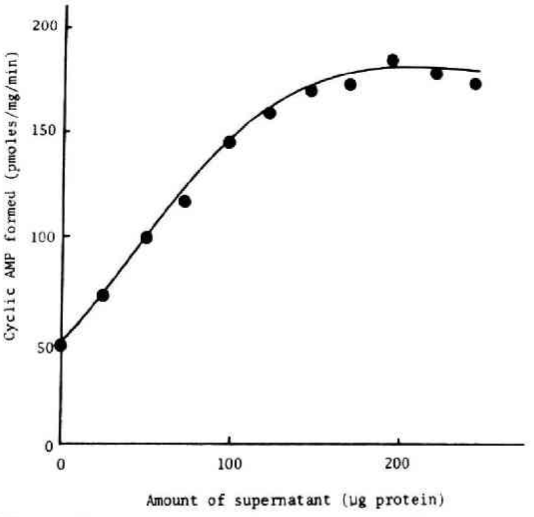


Fig. 4-1. Effects of supernatant concentration on adenylate cyclase activity in synaptosomes from rat cerebral cortex. The supernatant was obtained as described in Methods. The amount of supernatant varied from 0 to 250 μg of protein.

Table 4-1. Effect of Dialysis of Supernatant

Additions	Adenylate cyclase activity (pmoles cyclic AMP/min/mg protein)
None-control	39.3 ± 10.9
Supernatant	126.3 ± 8.7
〃 (dialyzed for 6 hr)	61.5 ± 11.4
〃 (dialyzed for 12 hr)	40.5 ± 3.8

The supernatant (130 μg protein) was obtained as described in Methods and then dialyzed for 6 hours (2 × 500 volumes) and 24 hours (4 × 500 volumes) versus 50 mM Tris-buffer (pH 7.4). Data were averages ± S.D. of 3 separate analyses.

determine the amount of protein of the supernatant so that linear part of the curve was used.

4-2. *Effect of Dialysis*

The effect of dialysis of the supernatant has been examined (Table 4-1). The supernatant was dialyzed for 6 hours at 4°C (2 × 500 volumes). Dialysis of the supernatant caused a marked loss of the stimulatory activity. Larger dialysis (24 hours, 4 × 500 volumes) of the supernatant resulted in a complete loss of the stimulatory activity.

4-3. *Chromatography on Sephadex G-25 Superfine*

The stimulatory substance in the supernatant was purified using chromatography on Sephadex G-25 superfine. Figure 4-2 depicts the elution profile of the lyophilized supernatant from the column. As seen in Fig. 4-2, the peak stimulated activity was eluted at 53 ml. Calibration of this column with glucagon (M.W. 3,350) and vitamin B_{12} (M.W. 1,350) as markers indicated that this elution volume corresponded to a

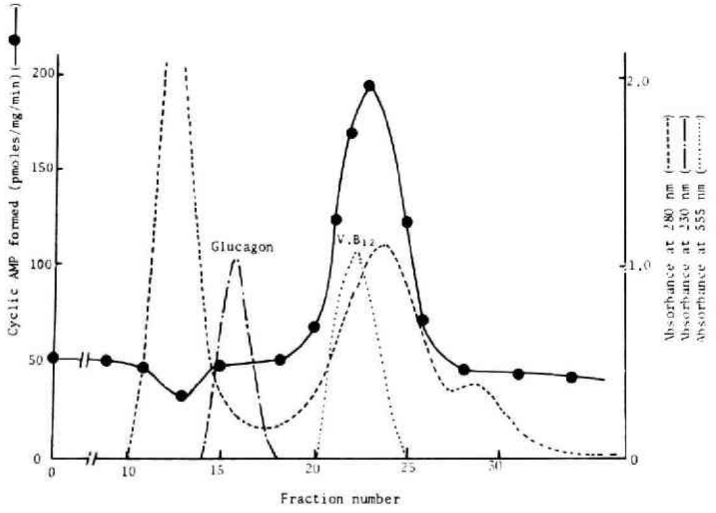


Fig. 4-2. Chromatography of adenylate cyclase stimulatory substance(s) on Sephadex G-25 superfine. Rat brain cerebral cortex was homogenized in five volumes of ice-cold 50 mM Tris-HCl buffer (pH 7.4). The homogenates were centrifuged; 1,000×g for 20 min, 10,000×g for 20 min and 105,000×g for 60 min. The resulting supernatant was lyopyilized and then applied to a column of Sephadex G-25 superfine (1.5×43 cm) equilibrated previously in the homogenization buffer. The column was eluted at 4°C with the same buffer in 2.5 ml fractions at a flow rate of 10 ml per hour. An aliquot (0.1 ml) of each fraction was assayed for its adility to activate the synaptosomal adenylate activity. The void volume of the column was 23 ml and the peak stimulated activity was eluted at 53 ml.

Table 4-2. Effect of Heat Treatment

Additions	Cyclic AMP formed (pmoles/mg of protein/min)
None	50.6± 3.3
Supernatant	105.1± 0.3
Boiled supernatant	384.1±30.9

The native and boiled supernatants were obtained as described in Methods. The amounts of the natvie and boiled supernatants were 150 μg and 130 μg of protein, respectively. Data were averages ± S.D. of 3 separate analyses.

molecular weight of less than 1,300.

4-4. *Effect of Heat Treatment*

When the supernatant was heated in a boiling water bath for 5 min and then, after 10-20 min at 0°C, added to the reaction mixture, the rate of cyclic AMP accumulation was much higher than by the native supernatant (Table 4-2). This finding indicates that the stimulatory substance(s) was heat-stable. The difference of cyclic AMP accumulation between the native supernatant and the boiled supernatant seems to be ascribable to the presence of cyclic AMP phosphodiesterase which involved in the

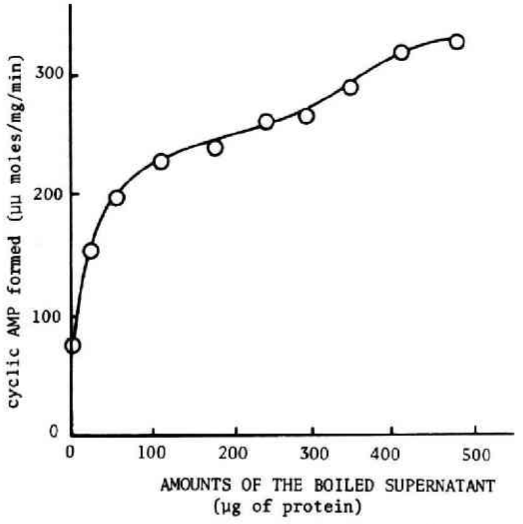


Fig. 4-3. Effect of the boiled supernatant concentration on the formation of cyclic AMP in synaptosomes. Each sample contained synaptosomes (0.2 mg of protein) as enzyme source.

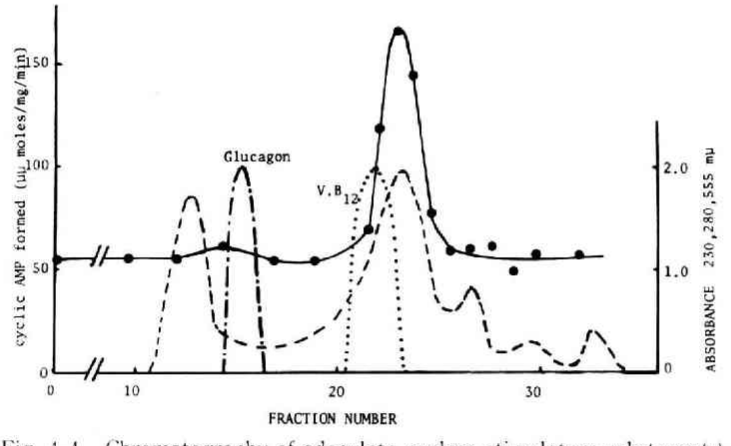


Fig. 4-4. Chromatography of adenylate cyclase stimulatory substance(s) on Sephadex G-25 superfine. The boiled supernatant was prepared by heating the 105,000 × g supernatant from rat brain shown in Fig. 4-2 in a boiling water bath and then centrifuged at 10,000 × g for 10 min to remove denatured proteins. This supernatant was lyophilized and then applied to a column of Sephadex G-25 superfine (1.5 × 43 cm) equilibrated previously in the homogenization buffer. The column was eluted at 4°C with same buffer in 2.5 ml fractions at a flow rate of 10 ml per hour. An aliquot (100 μl) of each fraction was assayed for its ability to activate the synaptosomal adenylate cyclase activity. The void volume of the column was 23 ml and the peak stimulated activity was eluted at 53 ml.

Table 4-3. Separation of the boiled supernatant by ultrafiltration (G-01T)

Additions	Cyclic AMP formed (pmoles/mg of protein/min)
None	63.5 ± 1.8
Boiled supernatant	322.3 ± 2.6
Filtrate of G-01T	67.6 ± 7.2

The boiled supernatant obtained as described in Methods, equivalent to 350 μg of protein, was added to each incubation medium. The boiled supernatant was filtrated with Ulvac ultrafiltration cell equipped with a G-01T membrane, and then an aliquot of the filtrate was assayed for its ability to stimulate synaptosomal adenylate cyclase. Data were averages ± S.D. of 3 separate analyses.

hydrolysis of cyclic AMP in the native supernatant.

When the concentration of the boiled supernatant increased as well as shown in Fig. 4-1, the extent of cyclic AMP accumulation was progressively greater (Fig. 4-3). When the boiled supernatant was fractionated by column chromatography (Sephadex G-25 superfine), similar peaks of stimulatory substance(s) were observed (Fig. 4-4).

4-5. *Ultrafiltration*

To determine more precise molecular weight of the stimulatory substance(s), ultrafiltration method (Blatt 1971) was used (Table 4-3). The boiled supernatant was filtrated with a Ulvac ultrafiltration cell equipped with a G-01T membrane, and then an aliquot (100 μl) of the filtrate was assayed for its ability to stimulate the adenylate cyclase activity. The results are shown in Table 4-3. This filtrate did not stimulate

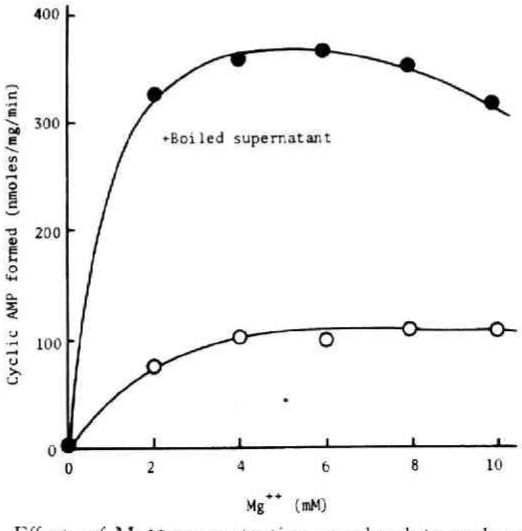


Fig. 4-5. Effects of Mg^{++} concentration on adenylate cyclase activity in synaptosomes in the absence (open symbols) and in the presence (closed symbols) of the boiled supernatant (0.3 mg of protein).

the enzymic activity at all, indicating that the stimulatory substance(s) has a molecular weight of more than 1,000. From the above results (Figs. 4-2, 4) and the present data (Table 4-3), the stimulatory substance(s) was estimated to have a molecular weight in the range of 1,000-1,300.

4-6. *Effect of* Mg^{++}

The effect of Mg^{++} concentration on the adenylate cyclase activity in synaptosomes in the absence and in the presence of the boiled supernatant is given in Fig. 4-5. When the concentration of ATP was fixed at 1 mM, the enzymic activity was not detected in the absence of Mg^{++} with and without the presence of the boiled supernatant whereas an increase in Mg^{++} concentration caused a progressive increase in the activity of adenylate cyclase with and without the presence of the boiled supernatant.

4-7. *Effect of Substrate (ATP)*

Assays of adenylate cyclase in synaptosomes were conducted in various ATP concentrations (Fig. 4-6). The calculation on the basis of the data obtained in the present studies revealed that Km values were 0.40×10^{-3} and 0.41×10^{-3}M in the absence and in the presence of the boiled supernatant, respectively, and Vmax values were 80 pmoles/min per mg protein and 400 pmoles/min per mg protein in the absence and in the presence of the boiled supernatant, respectively. Thus the boiled supernatant caused a five-fold increase of adenylate cyclase activity without significant influence on the Km values.

4-8. *Discussion*

From the results in this chapter, it was found that the stimulatory substance(s) in the supernatant was heat-stable, dialyzable, and had a molecular weight of about 1,200. However, at this stage, the detail nature of this substance(s) has not yet been clarified.

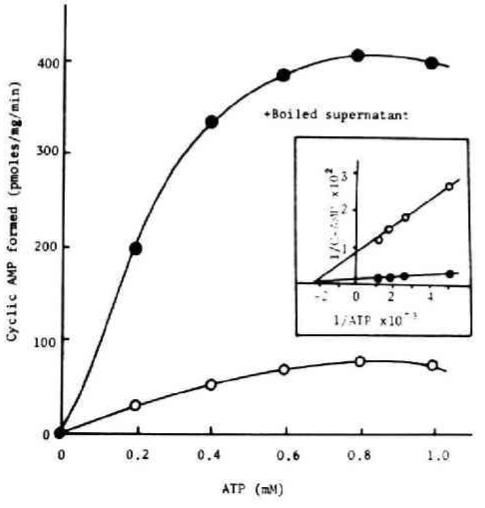


Fig. 4-6. Mechanism of the stimulatory effect of adenylate cyclase by the boiled supernatant (0.3 mg of protein). The effect of the boiled supernatant was studied by the double reciprocal plot (inset).

As can be seen from Fig. 4-5, adenylate cyclase in synaptosomes requires Mg^{++} for its activity and for its stimulation by the boiled supernatant. The basal activity of the enzyme was increased and then reached a plateau at Mg^{++} concentration of 4 mM and in the presence of the boiled supernatant the enzymic activity peaked at the same Mg^{++} concentration.

It is concluded that while the stimulatory effect of the boiled supernatant is due to an increase in Vmax value, there is no significant effect of the apparent affinity for substrate (ATP) (Fig. 4-6). The present results suggest that this stimulation is similar kinetically to the stimulations of fluoride (Bar et al. 1969) and hormone (Drummond et al. 1971). Although it was found the optimun concentration of ATP to be 0.8 mM, the significance of this value is difficult to asses since the isolated synaptosomes contain other enzymes which act on ATP, ADP, and AMP. These enzymes are ATPase, myokinase, ATP pyrophosphohydrolase and 5'-nucleotidase. Hence the actual concentration of ATP added to the synaptosomes is very rapidly lowered by the active ATPase and partially regenerated by myokinase. Birnbaumer and Rodbell (1969) attempted to circumvent this problem by adding ATP generating enzymes. However, Dunnick et al. (1972) have recently reported that the effect of a ATP regenerating system on adenylate cyclase is negligible. Accordingly, unless the ATP levels are actually measured at different time intervals, the true concentration of ATP remains unknown.

In many tissues the intracellular level of cyclic AMP is an important factor in the regulation of cell metabolism. In the absence of an external stimulus the concentration of bound and free cyclic AMP in various tissues are of the order of $10^{-7}-10^{-8}$M. This concentration is 1,000-10,000 times lower than that of ATP or 5'-AMP. The concentration of cyclic AMP in the cell is a function of the rates of its synthesis and degradation, which at any given times are determined by the relative activities of

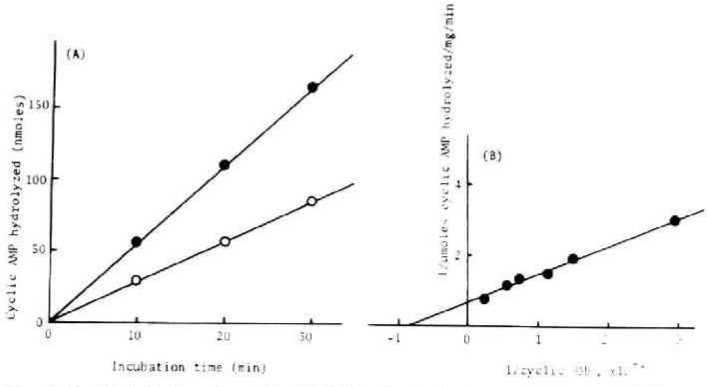


Fig. 4-7. (A) Rate of cyclic AMP hydrolysis in supernatant of the rat cerebral cortex. —●— : 93 μg protein concentration of the supernatant. —○— : 46 μg protein concentration of the supernatant. (B) Double-reciprocal plot of the hydrolysis of cyclic AMP by the supernatant from rat cerebral cortex. Substrate concentrations ranged from 33.75 μM to 360 μM for cyclic AMP.

adenylate cyclase and cyclic AMP phosphodiesterase. These activities are undoubtly influenced by a number of factors in addition to hormones, and very little is understood about their modes of action. Although cyclic AMP phosphodiesterase in the supernatant is several times higher than that of adenylate cyclase in synaptosomes as shown in Fig. 4-7 (A), addition of the supernatant to the adenylate cyclase assay system does not decrease the cyclic AMP levels, but increase it. This may occur because of sequestration of cyclic AMP in bound form with the receptor protein or by compartmentalization of cyclic AMP in a part of the cell inaccessible to cyclic AMP phosphodiesterase, and high Km value of cyclic AMP phosphodiesterase in the supernatant (130 μM) (Fig. 4-7B).

CHAPTER 5. SITE OF ACTION OF THE STIMULATORY SUBSTANCE(s)

5-1. *Effects of the Native and Boiled Supernatants on Brain ATPase*

In order to determine whether or not the boiled supernatant induced enhancement of cyclic AMP accumulation was due to increased availability of substrate (ATP) for adenylate cyclase, the effects of the native and the boiled supernatants on the brain ATPase activity in synaptosomes were investigated, and the results are shown in Table 5-1. As can be seen, the native and boiled supernatants inhibited ATPase activity by about 20%.

5-2. *Effects of the Boiled Supernatant on Cyclic AMP Phosphodiesterase*

To determine whether or not the boiled supernatant-induced stimulation of cyclic

Table 5-1. Effects of the basal and boiled supernatants on synaptosomal ATPase activity

Additions	ATPase activity (μmolesPO$_4$/mg per min)
None	5.21 ± 0.06
Native supernatant (366 μg protein)	4.28 ± 0.04*
Boiled supernatant (300 μg protein)	4.07 ± 0.05*

* Significantly different from control ($p<0.01$)
Data are averages ±S.D. of 3 separate analyses.

Table 5-2. Effect of the boiled supernatant on cyclic AMP phosphodiesterase activity

Preparations	Addition	Cyclic AMP hydrolyzed (nmoles/15 min)
Synaptosomes (100 μg protein)		40.4 ± 7.8
〃	+Boiled supernatant	65.1 ± 1.1*
Synaptosomes (200 μg protein)		66.3 ± 3.5
〃	+Boiled supernatant	102.5 ± 1.3*

* $p<0.001$ compared with each control.
The boiled supbrnatant obtained as described in Methods, equivalent 300 μg of protein, was added to each incubation medium. Data are averages ±S.D. of 3 separate analyses.

AMP accumulation occurred at the cyclic AMP phosphodiesterase step, the effects of the boiled supernatant on cyclic AMP phosphodiesterase activity in synaptosomes were investigated and the results are shown in Table 5-2. As can be seen, the synaptosomal cyclic AMP phosphodiesterase activity was also stimulated by the addition of the boiled supernatant about 1.5 fold, indicating that the stimulatory effect of the boiled supernatant on cyclic AMP accumulation was not the result of the inhibition of cyclic AMP phosphodiesterase activity.

5-3. *Effect of Sodium fluoride (NaF) on Adenylate Cyclase and ATPase*

In order to compare the concentration-response relationships between the inhibition of ATPase by sodium fluoride and the accumulation of cyclic AMP, the following experiments were carried out. As can be seen from Fig. 5-1, as the concentration of sodium fluoride increased, the extent of the inhibition of synaptosomal ATPase activity was proportionally greater, and, on the other hand, activation of synaptosomal adenylate cyclase by sodium fluoride was maximal at a concentration of about 5 mM ; higher concentrations of sodium fluoride reduced enzymic stimulation, indicating that there was no correlation between these two effects.

5-4. *Disussion*

As shown in chapter 2, 3, the native and boiled supernatants increased the accumulation of cyclic AMP about several fold when added to the adenylate cyclase assay system in synaptosomes from rat cerebral cortex. Adenylate cyclase from most tissues is not readily purified, primarily because it is membrane-bound and is relatively unstable. In the present studies, synaptosomes were utilized as an enzyme preparation. This preparation contains amount of ATPase and Cyclic AMP phosphodiesterase activities and whose presence therefore must be considered. Although other possibilities cannot be excluded completely at this stage, the ability of the boiled super-

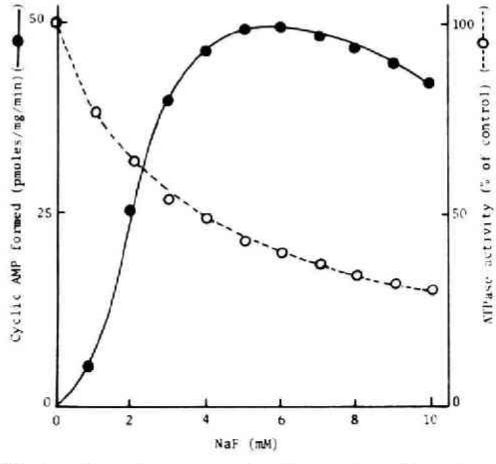


Fig. 5-1. Effects of various concentrations of sodium fluoride on adenylate cyclase activity (closed symbols) and ATPase activity (open symbols) in synaptosomes.

natant to stimulate cyclic AMP accumulation in synaptosomes may be due to the following three reasons: 1) direct stimulation of synaptosomal adenylate cyclase, 2) increase of the available substrate (ATP) by inhibition of the synaptosomal ATPase activity and 3) inhibition of synaptosomal cyclic AMP phosphodiesterase activity. As can be seen from Fig. 5-1, a study of the concentration response relationship between the inhibition of ATPase by sodium fluoride and stimulation of the accumulation of cyclic AMP revealed no correlation between the two effects, indicating that ATPase inhibition and cyclic AMP accumulation are not causally related. This suggests that the slight inhibition of ATPase activity by the boiled supernatant (Table 5-1) might not induce a marked increase of cyclic AMP accumulation. Schaefer et al. (1972, 1973, 1974, 1975) have previously reported the presence of the soluble inhibitory substance(s) on ATPase of rat brain. On the other hand, synaptosomal cyclic AMP phosphodiesterse was not inhibited but rather stimulated by the addition of the boiled supernatant as seen in Table 5-2. This stimulatory effect of the boiled supernatant on cyclic AMP phosphodiesterase was further investigated in the following chapter 7. The results shown in Table 5-2 indicate that the stimulatory effect of the boiled supernatant on cyclic AMP accumulation is apparently unrelated to the cyclic AMP phosphodiesterase activity. Although other possibilties cannot be excluded completely at this stage, it is conceivable from the above results that the mechanism responsible for the stimulation of accumulation of cyclic AMP by the stimulatory substance(s) in the supernatant might consist of an activation of adenylate cyclase. However, proof for the mechanism of this stimulatory effect on cyclic AMP formation in synaptosomes must await purifications of both enzyme (adenylate cyclase) and the stimulatory substance(s) in the supernatant.

CHAPTER 6. EFFECTS OF VARIOUS AGENTS ON SYNAPTOSOMAL ADENYLATE CYCLASE

6-1. *Effect of Norepinephrine*

As noted in the introduction, since it has been reported that the adenylate cyclase activity of brain tissue homogenates is not stimululated by catecholamine, the effect of

Table 6-1. The effects of norepinephrine and supernatant on adenylate cyclase activity in synaptosomes

Additions	Adenylate cyclase activity (pmoles cyclic AMP/min per mg protein)
None	38.1 ± 2.4
Supernatant	104.7 ± 3.3
Norepinephrine (100 μM)	33.4 ± 4.7
Supernatant + Norepinephrine	54.7 ± 5.0

The supernatant obtained as described in Methods, equivalent to 140 μg of protein, was added to each incubation medium. Norepinephrine was added at 100 μM. Data were averages ±S.D. of 3 separate analyses.

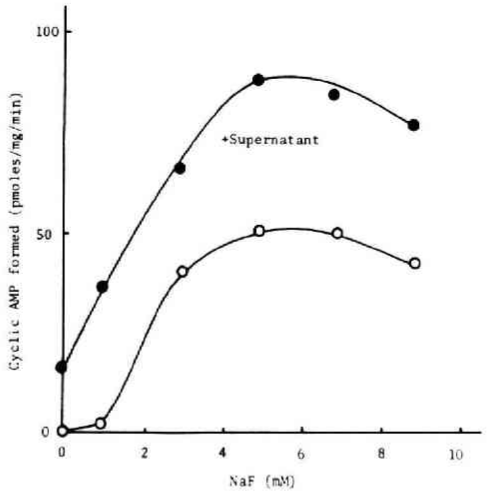


Fig. 6-1. Effects of the supernatant on the synaptosomal adenylate cyclase activity in the presence of various concentrations of sodium fluoride (NaF). Sodium fluoride was added to the assay mixyture at the concentrations indicated. The supernatant, equivalent 180 μg of protein, was obtained as described in Methods and was added to incubation medium.

norepinephrine on synaptosomal adenylate cyclase activity has been investigated in the absence and in the presence of the supernatant. As seen in Table 6-1, in contrast to the stimulatory effect of the supernatant, norepinephrine (100 μM) did not influence the basal enzyme activity significantly. On the other hand, the stimulatory effect of the supernatant was markedly reduced by the addition of norepinephrine.

6-2. *Effect of Sodium Fluoride*

Sodium fluoride is a well-known potent activator of adenylate cyclase in various tissue homogenates (Perkin and Moore 1971 ; Barr and Hechter 1969 ; Drummond et al. 1971 ; Weiss 1969 ; Menson et al 1973). In order to determine whether the sites of actions of the stimulatory substance(s) in the supernatant and sodium fluoride on the cyclic AMP accumulation are the same or not, the effect of the supernatant on the adenylate cyclase activity in the absence and in the presence of sodium fluoride was investigated. As seen in Fig. 6-1, the stimulatory substance(s) induced a further increase in the rate of the formation of cyclic AMP in the presence of sodium fluoride, indicating that the stimulatory snbstance(s) and sodium fluoride act at distinctly different sites in the adenylate cyclase system of rat brain synaptosomes.

6-3. *Role of Sulfhydryl Groups*

The effects of p-chloromercuribenzoate (p-CMB), which reacts with sulfhydryl groups, in various concentrations on the synaptosomal adenylate cyclase in the absence and in the presence of the boiled supernatant are given in Fig. 6-2. The basal enzyme activity was not affected by the addition of p-CMB at 10^{-6}M concentration but was inhibited by the addition of more than 10^{-5}M p-CMB. On the other hand,

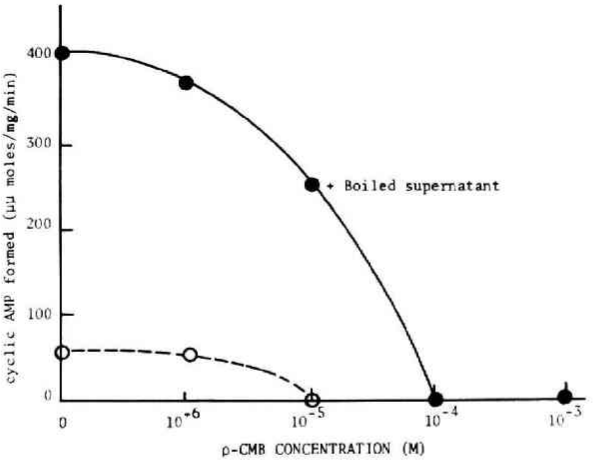


Fig. 6-2. Dose response curve showing the effect of p-CMB on the cyclic AMP formation in synaptosomes in the absence (open symbols) and in the presence (closed symbols) of the boiled supernatant (0.35 mg of protein).

Table 6-2. The effects of sulfhydryl reagents on the formation of cyclic AMP

	A*	B*	Cyclic AMP formed (pmoles/mg/min)
Synaptosomes			53.5
〃 +p-CMB (10^{-4}M)			0
〃 + 〃 +Boiled supernatant (boiled sup)			0
〃 +D.T.E. (10^{-3}M)			345.3
〃		+Boiled sup	148.5
Boiled sup		+Synaptosomes	328.1
Synaptosomes +p-CMB		+Boiled sup	0
〃 + 〃	+D.T.E.	+Bolied sup	0
Boiled sup. + 〃		+Synaptosomes	0
〃 + 〃	+D.T.E.	+Synaptosomes	288.3

The boiled supernatant obtained as described in Methods, equivalent to 304 μg of protein, was added to each incubation medium.

* Materials indicated each column were added to the flask ; A : 60 sec. B : 30 sec. prior to the addition of substrate (^{3}H-ATP). p-CMB : p-chloromercuribenzoate. D.T.E. : Dithioerythritol.

adenylate cyclase activity was profoundly stimulated by the addition of the boiled supernatant, however, this increased cyclic AMP formation was also gradually decreased by increasing the concentration of p-CMB. The differences between the concentrations of p-CMB required for the blockade of cyclic AMP formation in synaptosomes in the absence and in the presence of the boiled supernatant suggests that the boiled supernatant might have sulfhydryl groups, however, it is not clear at this stage whether or not the stimulatory substance(s) in the boiled supernatant does indeed

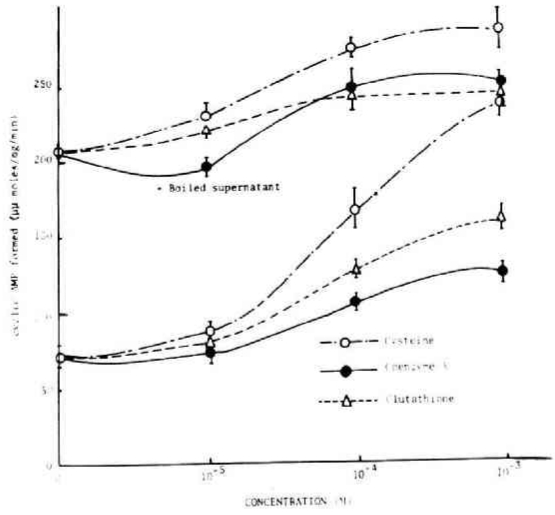


Fig. 6-3. Effects of varying concentrations of cysteine, glutathione and coenzyme A on the cyclic AMP formation in synaptosomes in the absence and in the presence of the boiled supernatant (0.2 mg of protein). —○—, Cysteine. ---△---, Glutathione. —●—, Coenzyme A.

have sulfhydryl groups, since the purification of this stimulatory substance(s) is still in progress.

To examine the more precise role of sulfhydryl groups of synaptosomal adenylate cyclase, the order of addition of synaptosomes (enzymes), p-CMB, the boiled supernatant and dithioerythritol, which maintains sulfhydryl groups, varied during preincubation and the results are shown in Table 6-2. As can be seen, when synaptosomes were added to the incubation medium after the addition of dithioerythritol (10^{-3}M), the enzymic activity was not inhibited by p-CMB (10^{-4}M). However, once sulfhydryl groups of synaptosomes had been occupied by p-CMB, the enzymic activity was not restored by the addition of dithioerythritol.

As can be seen from Table 6-2, synaptosomal adenylate cyclse was sensitive to dithioerythritol and thus the following experiments were carried out. When sulfhydryl compounds such as cysteine, glutathione and coenzyme A were added to the assay system, all three sulfhydryl compounds increased the enzymic activity in both the absence and presence of the boiled supernatant as shown in Fig. 6-3. Of the three compounds, cysteine was the most effective.

6-4. *Effects of Adenine Nucleotides*

The effects of adenine, adenosine and adenosine monophosphate (AMP) on the synaptosomal adenylate cyclase in the absence and in the presence of the boiled supernatant are shown in Fig. 6-4. Higher concentrations of AMP (10^{-3}M) inhibited the basal enzymic activity. The increased cyclic AMP formation by the boiled supernatant was reduced by higher oncentrations of adenosine and AMP.

6-5. *Effect of 1, 2-bis-(2-dicarboxymetiylaminoethoxy)ethane (EGTA)*

The effect of the inclusion of various concentrations of EGTA in the enzyme reaction mixture in the absence and in the presence of the boiled supernatant is shown in Fig. 6-5. As illustrated in this figure, maximal stimulatory effects were obtained when the concentrations of EGTA were 10^{-4}M and 5×10^{-5}M in the absence and in the presence of the boiled supernatant, respectively.

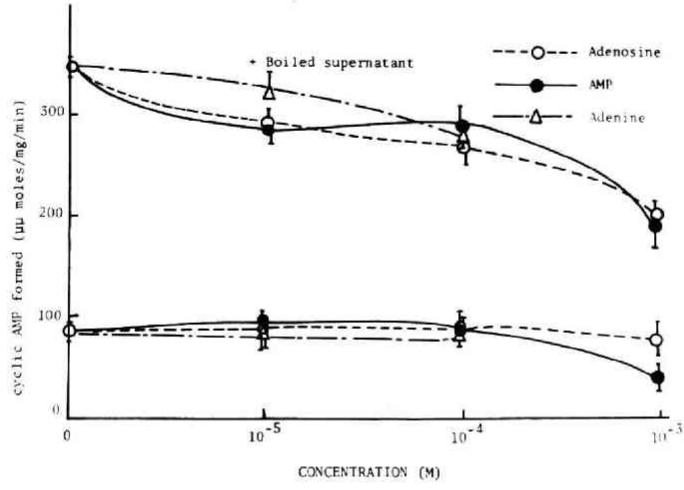


Fig. 6-4. Effects of varying concentrations of adenine nucleotides on the formation of cyclic AMP in synaptosomes in the absence and in the presence of the boiled supernatant (0.25 mg of protein). —△—, Adenine. ···○···, Adenosine. —●—, AMP.

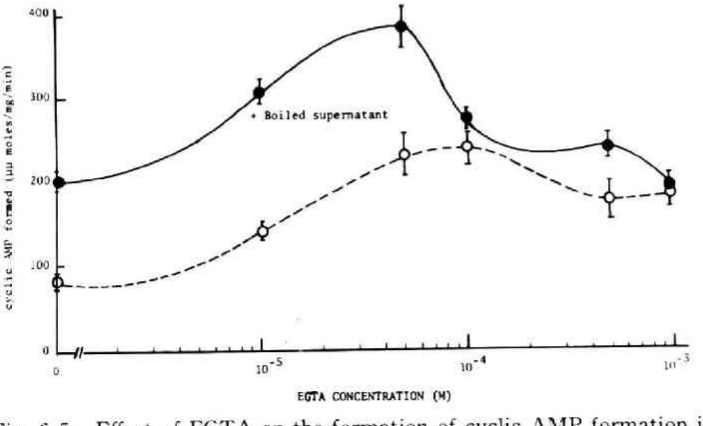


Fig. 6-5. Effect of EGTA on the formation of cyclic AMP formation in synaptosomes in the absence (open symbols) and in the presence (closed symbols) of the boiled supernatant (0.2 mg of protein). Varying concentrations of EGTA were included in the usual incubation medium. Each value represents the mean of 3 determinations with standard derivation.

Table 6-3. Effects of products of ATP breakdown on the formation of cyclic AMP in synaptosomes

Additions	Cyclic AMP formed (pmoles/mg of protein/min)
None	62.2
Boiled supernatant	457.1
ADP (10^{-4}M)	73.6
Boiled sup + ADP (10^{-4}M)	321.8
ADP (10^{-3}M)	26.4
Boiled sup + ADP (10^{-3}M)	180.6
Pi (10^{-4}M)	73.4
Boiled sup + Pi (10^{-4}M)	309.2
Pi (10^{-3}M)	61.8
Boiled sup + Pi (10^{-3}M)	256.7

The boiled supernatant obtained as described in Methods, equivalent to 304 μg of protein, was added to each incubation medium.

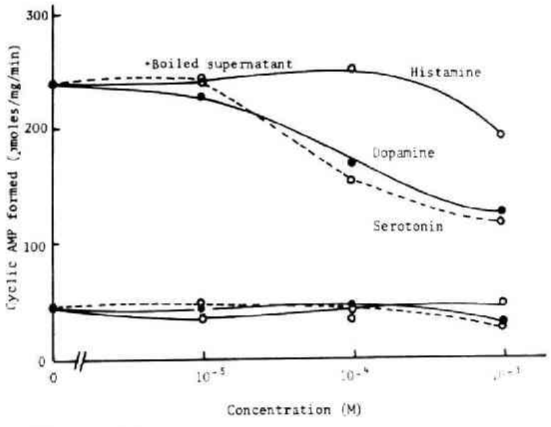


Fig. 6-6. Effects of dopamine and serotonin on synaptosomal adenylate cyclase in the absence and in the presence of the boiled supernatant (0.25 mg of protein). —●— Dopamine. —○— Histamine. ···○··· Serotonin.

6-6. *Effects of Adenosine Diphosphate (ADP) and Phosphoric Acid (Pi)*

The effects of ADP and Pi on the synaptosomal adenylate cyclase were investigated. As can be seen in Table 6-3, in the absence of the boiled supernatant ADP (10^{-4}M) and Pi (10^{-4}M) gave a slight stimulation, ADP (10^{-3}M) gave an appreciable inhibition but Pi (10^{-3}M) did not effect significantly to the enzymic activity. Stimulation of cyclic AMP accumulation by the boiled supernatant was gradually decreased by increasing the concentration of either ADP and Pi.

6-7. *Effects of Dopamine, Histamine and Serotonin*

The effects of neurotransmitters such as dopamine, histamine and serotonin on the synaptosomal adenylate cyclase are shown in Fig. 6-6. The basal enzymic activ-

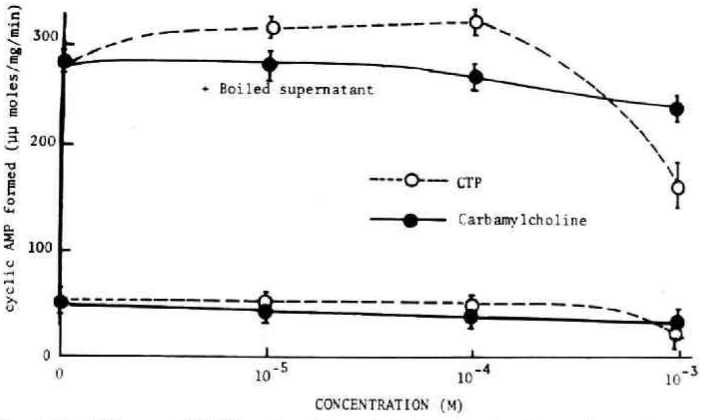


Fig. 6-7. Effects of GTP and carbamylcholine on the formation of cyclic AMP in synaptosomes in the absence and in the presence of the boiled supernatant (0.25 mg of protein). Each value represents the mean of 2 determinations on one preparation.

ity was not affected by the addition of such neurotransmitters but the boiled supernatant-stimulated enzymic activity was reduced by higher concentrations of dopamine and serotonin.

6-8. *Effect of Guanosine Triphosphate (GTP)*

The effects of varying concentrations of GTP on the synaptosomal adenylate cyclase in the absence and in the presence of the boiled supernatant were investigated and the results are shown in Fig. 6-7. GTP seems to have little effect on the basal and the boiled supernatant-stimulated cyclic AMP formation in synaptosomes as well as in the preparation of horse parathroid gland (Matsuzaki et al. 1972).

6-9. *Effect of Carbamylcholine*

The effect of carbamylcholine on the synaptosomal adenylate cyclase in the absence and in the presence of the boiled supernatant is given in Fig. 6-7. As can be seen, carbamylcholine did not affect the basal and the boiled supernatant-stimulated cyclic AMP formation at all.

6-10. *Discussion*

Previously, synaptosomes have been difined by Whittaker (1965) and Whittaker and Greengard (1971) as detached nerve endings, usually including postsynaptic membranes and intersynaptic structures. During the process of isolation, these pinched off nerve terminals seal, thus maintaining an inner biochemical composition which closely resembles that of the synaptic ending in the living animal. This finding had led many investigators to study the in vitro mechanism of synaptosomes, in order to help understanding of the role played by synaptic structures in the dynamic processes of the nervous system. One metabolic function of synaptosomes, the ability to incorporate amino acid into protein in vitro, has been the subject of much interest amoung neurobiologists.

Since the original report by Morgan and Austin (1968), many papers have ap-

peared dealing with the in vitro incorporation of radioactive precursors into macromolecules of synaptosomal preparations (Gordon and Deanin 1968 ; Autilio et al. 1968 ; Morgan and Austin 1968, 1969 ; Appel et al. 1969 ; Bosmann and Hemsworth 1970 ; Goldberg 1971 ; Cotman and Taylor 1971 ; Bridger et al. 1971 ; Gambetti et al. 1970 ; Hernandez et al. 1971). In addition, studies on the axonal transport of protein (Droz and Barondes 1969) have provided indirect evidence for local protein synthesis in nerve endings. From these data, brain synaptosomes seem to be able to serve an excellent in vitro model for analyzing the effects of agent on these uptake mechanism.

From the electron microscopic observation shown in Fig. 3-2, synaptosomes appear to be presynaptic nerve endings which are detached from their axons and postsynaptic attachments and seal off to form small particles when the brain tissue is homogenized. As noted previously, these particles contain mitochondria and synaptic vesicles and retain many of the morphological and chemical characteristics of the intact synaptic terminal. These facts led us to pursure the properties of the synaptosomal adenylate cyclase.

Recently, it has been reported that methylmercury is a potent inhibitor of adenylate cyclase in rat liver plasma membranes (Storm and Gunsalus 1974). The results in Fig. 6-2 showing that the synaptosomal adenylate cyclase from the rat brain cerebral cortex was inhibited by the sulfhydryl reagent such as p-CMB suggest that organomercurials may react well with protein sulfhydryl groups of synaptosomal plasma membranes as those of liver plasma membranes. It has been reported, however, that sulfhydryl groups were required for adenylate cyclase activity of rat liver plasma membranes but were not required for the stimulation of this activity by sodium fluoride or ACTH (Kelly and Koritz 1971). The cyclic AMP accumulation in the presence of both the boiled supernatant and 10^{-5}M p-CMB shown in Fig. 6-2 appears to be caused by the interaction between the remaining sulfhydryl groups of synaptosomes which had not reacted with p-CMB and a stimulatory substance(s) in the boiled supernatant. Once these sulfhydryl groups of synaptosomes had been lost as a result of reaction with p-CMB, the enzymic activity was not restored by the addition of dithioerythritol as can be seen from Table 6-2. These results indicate that sulfhydryl groups are required for the basal enzyme activity and for its stimulation by the boiled supernatant. This obserration supports the hypothesis that the sensitive sulfhydryl groups(s) may be associated with the catalytic subunit of adenylate cyclase. It has been suggested by Storm and Dolginow (1973) that the reactivity of the sulfhydryl group(s) of liver plasma membranes is pronouncely stimulated by glucagon and its binding may promote conformational changes within the adenylate cyclase system resulting in either increased or enhanced nucleophilicity of the crucial sulfhydryl group(s).

Divalent cations play a crucial and to some extent not yet well understood role in regulation of adenylate cyclase activity. Thus Sutherland and co-workers (Sutherland and Rall 1958, 1962 ; Rall and Sutherland 1962 ; Murad et al. 1962), the discoverers of adenylate cyclase, first pointed out that this enzyme had an absolute requirment

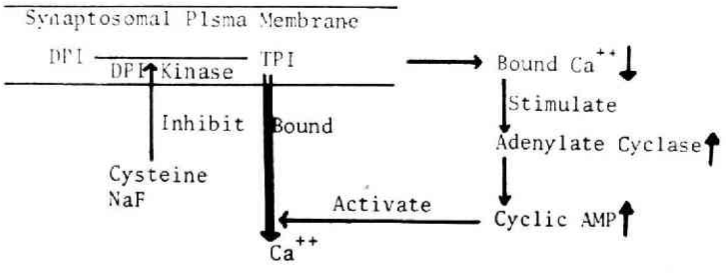
for Mg^{++}.

On the other hand, several inconclusive results concerning the effect of Ca^{++} on adenylate cyclase have been reported (Williams et al 1969; Taunton et al. 1969; Robison et al. 1970; Bradham et al. 1970; Bradham 1972; Hungen and Roberts 1973). The results in Fig. 6-5 show that EGTA, which is a specific chelator for Ca^{++}, caused a stimulation of the enzymic activity with and without the boiled supernatant. This stimulatory effect has not been adequately explained. Ca^{++} also inhibits adenylate cyclase in other tissues: fat, heart and adrenal tissues (Birnbaumer et al. 1969; Drummond and Duncan 1970; Lefkowitz et al. 1970) but to a lesser extent. Birnbaumer et al. (1969) suggested that calcium and magnesium compete for a putative allosteric site of the adenylate cyclase enzyme system in fat cell membrane. The data obtained in the present studies that the increase of synaptosomal adenylate cyclase in response to EGTA in the presence of 3.3 mM Mg^{++}, at which concentration the enzyme is approaching saturation with regard to Mg^{++} suggest that in this tissue Ca^{++} is exerting its effect by competing Mg^{++} for occupancy of an allosteric site. It therefore seems certain that Ca^{++} plays a significant role in controlling the adenylate cyclase activity in synaptosomes.

Although the inhibitory effect of adenosine on adenylate cyclase of rat liver has previously been reported (Moriwaki and Foa 1970), such an effect of adenosine on synaptosomal adenylate cyclase was not observed in the present studies (Fig. 6-4). The inhibitory effects were seen by the addition of adenine nucleotide (AMP, ADP) at higher concentrations, however, these actions do not seem to be physiological.

Recently, Harwood and Rodbel (1973) have reported that the loss of hormone sensitivity in the preparation of fat cell ghost is probably due to fluoride ion. In the present studies, since synaptosomal adenylate cyclase activity was not detected in the absence of fluoride as seen in Fig. 6-1, sodium fluoride was customary added to the incubation medium. Under these conditions, as seen from Table 6-1 and Fig. 6-6, no stimulatory effect by catecholamines such as norepinephrine, dopamine and serotonin was observed in the absence and in the presence of either the native supernatant or boiled supernatant. These results suggest that the process of cell disruption may cause a dissociation of the functional relationship between the receptor and the catalytic composition of brain adenylate cyclase.

Purine nucleotide triphosphates have been shown to be necessary for the expression of hormone stimulation, not only in the glucagon-sensitive system from liver parechymal cell (Birnbaumer et al. 1972), but also in the glucagon-sensitive system from pancreatic cell (Golfine and Birnbaumer 1972), and in prostaglandin-sensitive system from human plateletes (Krishna et al. 1972). It is, therefore, of prime importance to test the hypothesis the purine nucleotide-dependent step (notable GTP dependent) might be an invariant feature of the hormone-sensitive adenylate cyclase system, possibly acting at the step that couples hormone-receptor interaction to enzyme activation. The effects of varying concentrations of GTP on synaptosomal adenyate cyclase in the absence and presence of the boiled supernatant were investi-



DPI: Diphosphoinositide
TPI: Triphosphoinositide

Fig. 6-8 Hypothetical scheme of the relationship between the increase of cyclic AMP formation, Ca^{++}, sodium fluoride and cysteine in synaptosomes.

gated in order to examine the interaction of GTP and the stimulatory substance(s) in the boiled supernatant (Fig. 6-7). As can be seen, GTP did not significantly affect the enzymic activity in either the absence or presence of the boiled supernatant, suggesting that there is no interaction between the two. These results are at variance with those previously reported concerning GTP or $G_{pp}(NH)_p$ activation of basal adenylate cyclase preparations from rat liver or fat plasma membranes (Rodbell et al. 1971; Londos et al. 1974) and mouse neuroblastoma × glioma hybrid cell (Sharma et al. 1975). Such a variance may be due to the different assay conditions of the incubation system or to the different enzyme preparations.

It has been previously reported that synaptosomal plasma membrane differs from most other plasma membranes as described below. Erythrocyte ghost (Dodge and Phillips 1967), and plasma membranes from plateletes (Marcus et al. 1969), liver (Pfleger et al. 1965; Dod and Gray 1968; Ray et al. 1969) and L-cells (Weistein et al. 1968) all have higher cholesterol/phospholipid molar ratios and contain much greater amounts of sphingomyeline. In general, other plasma membranes seem to be characterized by a low content of glycolipids, but in the case of the synaptosomal plasma membranes, there are high levels of gangliosides and triphosphoinositide (Lapetina et al. 1967; Breckenridge et al. 1972; Hayashi 1975). Accordingly, in view of the present results and the following results by other investigators shown below, the following tentative model for the system which regulates cyclic AMP levels in synaptosomes is proposed as shown in Fig. 6-8. The following results have earlier been reported: (1) Both cysteine and sodium fluoride inhibit diphosphoinositide (DPI) kinase activity (Kay 1968) and stimulate cyclic AMP formation in synaptosomes (in this thesis). (2) Ca^{++} inhibit adenylate cyclase activity or EGTA stimulate adenylate cyclase activity (Hungen and Roberts 1973; Izumi and Ozawa 1975). (3) Triphosphoinositide (TPI) binds with Ca^{++} (Dawson 1965). (4) Cyclic AMP activates the binding of Ca^{++} with plasma membrane (Shatz 1972). (5) A number of amounts of TPI and DPI exists in the brain (Dawson and Diffmer 1961). (6) Ca^{++} in excitable membrane has a physiologically important function: the ion-permerable resting state is maintained by the

binding of Ca^{++} on it, by the liberation of Ca^{++} the polarity of the membrane component changes and becomes ion-permeable, and this process could be the initial step in excitaion (Tasaki 1968). (7) The dissociating effect of acetylcholime on TPI-protein-Ca^{++} may be involvled in excitation process of the synaptic membrane (Hayashi and Katagiri 1974). If this postulation is true, it seems reasonable that the increase of cyclic AMP formation by cysteine and sodium fluoride are mediated via their effects on DPI kinase. On the other hand, EGTA decreases the membrane bound Ca^{++} levels, and then produces the increase of cyclic AMP accumulation. However, it remains unknown where the stimulatory substance(s) in the supernatant acts.

Chapter 7 Stimulation of Synaptosomal Cyclic AMP Phosphodiesterase by the Boiled Supernatant

7-1. *Separation of the Stimulatory Substance(s) on Sephadex G-25 Superfine*

As previosuly mentioned in chapter 3, the supernatant contained the soluble stim-

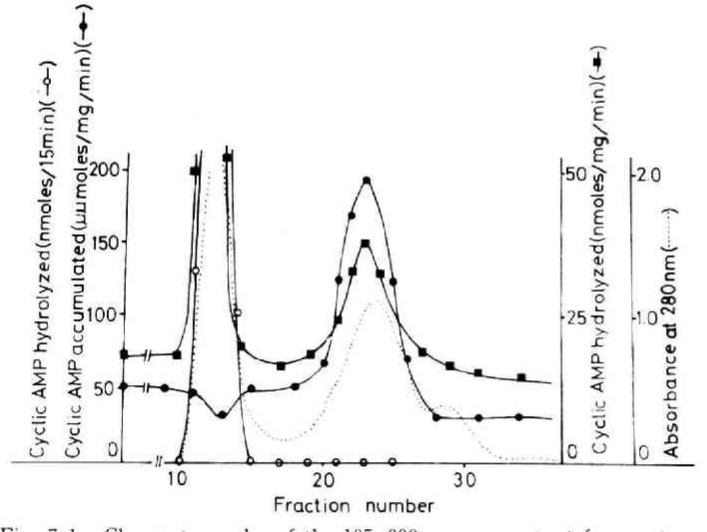


Fig. 7-1. Chromatography of the 105,000×g supernatant from rat cerebral cortex on Sephadex G-25 superfine. Rat brain cerebral cortex was homogenized in five volumes of ice-cold 10 mM Tris-HCl buffer (pH 7.4). The homogenates was centrifuged; 1,000×g for 20 min. 10,000×g for 20 min and 105,000×g for 60 min. The resulting supernatant was lyophilized and then applied to a column of Sephadex G-25 superfine (1.5×43 cm) equilibrated previously in 50 mMTris-HCl buffer (pH 7.4). The column was elued with same buffer in 2.5 ml fractions at a flow rate of 10 ml per hour. An aliquot (100 μl) of each fraction was assayed for its cyclic AMP phosphodiesterase activity (—○—) and its ability to activate the synaptosomal adenylate cyclase (●) or synaptosomal cyclic AMP phosphodiesterase activities (—■—). The protein content of synaptosomes was 200 μg in each asch assay. Column buffer alone has no effects on adenylate cyclase and cyclic AMP Phosphodiesterase activities.

ulatory substance(s) which increased the accumulation of cyclic AMP in synaptosomes from rat brain cerebral cortex. The finding prompted us to determine whether or not this accumulation of cyclic AMP resulted from an inhibition of cyclic AMP phosphodiesterase in synaptosomes. Accordingly, the effects of the boiled supernatant on this enzyme have been investigated. As described earlier in chapter 5, synaptosomal cyclic AMP phosphodiesterase was also activated by the boiled supernatant. Hence the following experiments were carried out. As can be seen in Fig. 7-1, when aliquots (100 μl) of the Sephadex G-25 column fraction were examined for the ability to activate the synaptosomal cyclic AMP phosphodiesterase as well as in case of the adenylate cyclase shown in Fig. 4-2, the coincident peaks of acitivating substance(s) were found (fraction 23) for two enzyme activities. The activity of first peak fraction (fraction 12-14) is likely to be originated from cyclic AMP phosphodiesterase contained in the supernatant, because this corresponding activity was not observed when the boiled supernatant was fractionated.

7-2. *Effects of Sodium Fluoride on Adenylate Cyclase and Cyclic AMP Phosphodiesterase*

In order to examine the relation of the stimulatory effects between adenylate cyclase and cyclic AMP phosphodiesterase, sodium fluoride, a potent activator of adenylate cyclase in tissue homogenates (Birnbaumer et al. 1969 ; Perkins and Moore 1971 ; Drummond et al. 1971), was added to each incubation medium, and then both enzyme activities were assayed. As shown in Fig. 7-2, it was found that a plot of adenylate cyclase activity to sodium fluoride concentration in synaptosomes was sigmoidal in nature in accordance with the observations of Birnbaumer et al. (1969) for rat fat cell ghost and of Perkins and Moore (1971) for rat cerebral cortex homogenates. However, the addition of varying concentrations of sodium fluoride to a cyclic

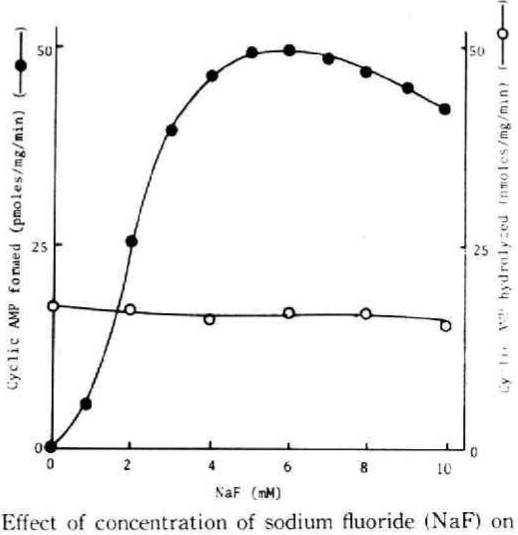


Fig. 7-2. Effect of concentration of sodium fluoride (NaF) on adenylate (—●—) and cyclic AMP phosphodiesterase activities (—○—) in synaptosomes from rat cerebral cortex. The protein content of synaptosomes was 200 μg in each assay.

Table 7-1. Effect of the stimulatory factor(s) on soluble supernatant and synaptosomal cyclic AMP phosphodiesterase activity

Enzyme preparations	Control	With stimulatory factor(s)
Supernatant	42.8 ± 5.1	39.8 ± 4.0
Synaptosomes	46.6 ± 5.1	84.1 ± 7.0*

Rat cerebral cortex was homogenized in 50 mM Tris-HCl buffer (pH 7.4). The homogenate was centrifuged at 1,000 × g for 20 min, 10,000 × g for 20 min and 105,000 × g for 60 min. The 105,000 × g supernatant was dialyzed extensively against the same buffer. An aliquot of this supernatant fluid was used as a source of cyclic AMP phosphodiesteaset The synaptosomes were prepared as described in the Methods. One-tenth ml of the stimulatory fraction (23 fraction) from a Sephadex G-25 superfine shown in Fig. 7-1 was added to the enzyme system as the stimulatory factor. Protein concentrations in μg per 0.5 ml of the reaction mixture : supernatant, 30 ; synaptosomes, 220. Activity is expressed as mμ moles of cyclic AMP hydrolyzed per 15 min. The values shown are the average results of single experiment carried out in triplicate. The ranges of values are represented by average deviations.

* The addition of the stimulatory factor significantly increased cyclic AMP phosphodiesterase activity of the synaptosomes ($p < 0.01$).

Table 7-2. Effects of various concentrations of imidazole on soluble supernatant and synaptosomal cyclic AMP phosphodiesterase activity

Addition Imidazole (mM)	Supernatant	Synaptosomes
0	35.3	54.9
5	40.6	52.8
10	41.5	55.1
20	51.7	77.1
40	54.9	82.4
80	44.6	73.7

The enzyme preparations of the supernatant and the synaptosomes were prepared as described in Table 7-1 and Methods, respectively. Protein concentrations in μg per 0.5 ml of the reaction mixture : supernatant, 30 ; synaptosomes 220. Activity is expressed as mμ moles of cyclic AMP hydrolyzed per 15 min. The solution of imidazole was adjusted to pH 7.4 with acetic acid.

AMP phosphodiesterase assay system did not affect the enzymic activity at all, suggesting that there is no relationship between the stimulation of adenylate cyclase and that of cyclic AMP phosphodiesterase.

7-3. *Effect of Imidazole*

It has been reported that the brain cyclic AMP phosphodiesterase is distributed in both soluble and particulate fractions (Robertis et al. 1967 ; Cheung and Salganicoff 1967). Although the relationship between these two enzymes is not fully understood, a comparative study of the effects of the stimulatory substance(s) in the supernatant

and imidazole on cyclic AMP phosphodiesterase in the both soluble and synaptosomal enzymes was carried out. As can be seen from Table 7-1, in contrast to membrane -bound cyclic AMP phosphodiesterase, the enzyme in the soluble supernatant was not stimulated by the addition of the stimulatory fraction. However, the addition of imidazole, an activator for cyclic AMP phosphodiesterase (Butcher and Sutherland 1962), stimulated the enzyme activity in both soluble and synaptosomal enzymes (Table 7-2). These results indicate that the mode of action of this stimulatory substance(s) on the synaptosomal cyclic AMP phosphodiesterase is different in some manner from that of imidazole. These results also suggest that this stimulatory substance(s) would be different from those obtained by various investigators (Kakiuchi and Yamazaki 1970 ; Cheung 1970, 1971 ; Teo et al. 1973) in molecular weight and the specificity on the membrane-bound cyclic AMP phosphodiesterase. However, it cannot be concluded at this stage whether the stimulatory substance(s) of adenylate cyclase and cyclic AMP phosphodiesterase are the same or not.

Chapter 8. Conclusion Discussion

There is evidence for a cellular localization of the catecholamines such as norepinephrine, dopamine and 5-hydroxytryptamine within specific neuron systems of the brains of laboratory animals (Carlsson et al. 1962 ; Falck et al. 1962). The distribution of the bodies and terminals of monoamine nerve cells is known in detail for the rat, and the presence of these cells had been documented also in other species (Dalstrom and Fuxe 1964). Figure 8-1 shows that the cerebral cortex of the rat contains a number of the nervous cells. Considerable amounts of catecholamines have been shown to present in the rat brain (Karki et al. 1962 ; Pletscher et al. 1963 ; Barchs and Freedman 1963 ; Hansson et al. 1964 ; Baird and Lewis 1964). However, the functional significance of the various monoamine neuron system, for instance their involvement in the mechanism of action of psychotropic drugs, is under study.

Brain tissue contains extremly high levels of adenylate cyclase, phosphodiesterase and protein kinase. The level varys in different brain regions ; it is particulary high in grey matter and low in white matter. In homogenates of brain tissue, high levels of adenylate cyclase, phosphodiesterase, protein kinase and phosphoprotein phosphatase have been found in particulate fractions generally referred to as "synaptosomes" (Robertis et al. 1967 ; Weiss and Costa 1968 ; Maeno et al. 1971 ; Maeno and Greengard 1972). As shown in Fig. 3-2, these consist of piched-off nerve-endings in the form of resealed entities containing mitochondria and transmitter storage vesicles, along with an adhering postsynaptic membrane. Thus, all the components of the cyclic AMP system are present as part of the synaptic complex.

However, as noted previously, the adenylate cyclase in homogenates of brain tissue hardly retains the receptor-mediated regulation of activity which is always seen in cell-free preparations from other tissues. Thus, although biogenic amines, adenosine and prostaglandin elicit significant accumulations of cyclic AMP in intact cells

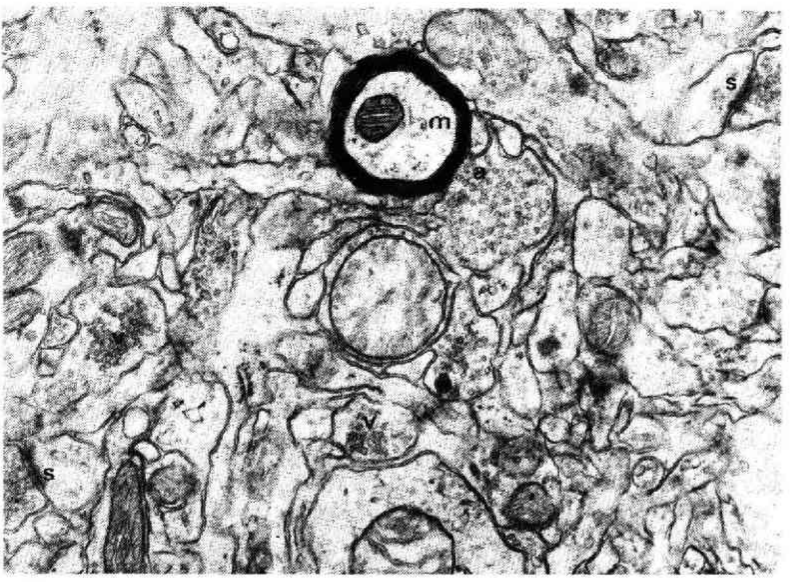


Fig. 8-1. Electron micrograph from rat cerebral cortex. m : myelinated axon, a : unmyelinated axon ; v : synaptic vesicle ; s : thickened apposed membrane of post-synaptic processes. × 25,000.

of brain slices, they have either no effect or only small effects on the activity of adenylate cyclase in homogenates of brain tissues (Sattin and Rall 1970 ; Klainer et al. 1962 ; William et al. 1969 ; Rall and Sattin 1970 ; McCune et al. 1971 ; Drummond et al. 1971). The magnitude of the small effects of such agents on adenylate cyclase in homogenates is dependent upon the methods of homogenization and assay, probably due to the lack of direct relationship of results obtained by different groups of investigators. In view of the present problems in retaining hormonal responsiveness of adenylate cyclase in homogenates, it would appear premature to attempt to difine the loci of hormone responsive systems present in the intact tissue by classical fractionation techniques.

More recently, homogenization and fractionation techniques which afforded a cell-free particulate preparation from brain tissue that retains a hormone-responsive cyclic AMP-generating system have been reported by Chasin et al. (1974). Particularly, they used a ^{3}H-adenine prelabelling technique and prepared a homogenates from cerebellum, cerebral cortex and hyppocampus in Krebs-Ringer buffer. This method enabled them to formulate a model of a cell-free, vesicular, hormonally responsive adenylate cyclase preparation from brain. However, in the present studies, the effects of the supernatant on adenylate cyclase cyclase in synaptosomal preparation have not been investigated using this method.

On the other hand, an increasing amount of evidence suggests that cyclic AMP may be involved in the regulation of metabolism and function in nervous tissuls (Greengard and Costa 1970). Certain data suggest that cyclic AMP may be intimately

associated with the molecular events underlying the process of synaptic transmission. For instance, evidence has been presented that exogenous cyclic AMP can mimic the inhibitory effect of norepinephrine on cerebellar Purkinje cells (Siggins et al. 1971). Moreover, synaptic activity in autonomic ganglia caused a several-fold increase in the amount of cyclic AMP in the ganglia in brief periods, which occurred largely, if not entirely, in postsynaptic cells (McAfee et al. 1970). Greengard et al. have previously suggested that this increase in cyclic AMP may modify the excitability of certain postsynaptic cells by the following chain of event (McAfee et al. 1970 ; Greengard and Kuo 1970) : (i) activation of a protein kinase by cyclic AMP, (ii) phosphorylation of the synaptic membrane by the kinase, (iii) change of ionic permeability of the membrane, (iv) change of membrane potential, and (v) change of excitability. Although this scheme provides a plausible molecular mechanism for prolong modulation of synaptic transmission either by facilitation or by inhibition, there are several objections to met before such a hypothesis can be adopted. For example, contrary to the above hypothesis, it has also been postulated by Rasmussen and Tenenhouse (1968) that as cyclic AMP is a weaker calcium ion chelating agent than is adenosine triphosphate (ATP), the conversion of membrane bound ATP to cyclic AMP releases Ca^{++} which is complexing with other membrane constituents such as phospholipids. And, as results, the membrane becomes permeable to Ca^{++}. The involvement of Ca^{++} in process such as catecholamine release and muscle contraction is well known ; so it seems that simply the conversion of ATP to cyclic AMP may have important physiological consequences.

As shown in this thesis, the supernatant from rat cerebral cortex contains a stimuiatory substance(s) which increases the accumulation of cyclic AMP in synaptosomes. However, at this stage a reasonable explanation for the physiological significance of this substance(s) cannot be offered.

The presence of low molecular factors concerning the regulatory factors such as an inhibitory factor of brain ATPase (Schaefer et al. 1972, 1973, 1974, 1975 ; Izumi et al. 1976a), endogenous inhibitors of dopamine-β-hydroxylase (Duch and Kirshner 1971 ; Izumi et al. 1975c ; Oyama et al. 1975a, 1976), a relaxing factor of smooth muscle (Florey 1953 ; Florey and Mclennan 1955 ; Suzuki et al. 1971), a collagen stimulatory factor (McGee et al. 1973) has earlier been reported. However, the both characterization and purification of these factors have not yet been completed, and their physiological functions are still the subject of controversy.

In view of the similar properties (heat-stable, dialyzable and probably sulfhydryl compounds) amoung the factors of adenylate cyclase (Izumi and Ozawa 1975), of dopamine-β-hydroxylase (Izumi et al. 1975b) and of ATPase (Izumi et al. 1976a), it is possible to speculate that these factors may be identical. However, it seems to be premature to draw a definite conclusion, because these have not yet been purified completely. On the other hand, since both enzymes, adenylate cyclase and ATPase, are membrane-bound enzymes and since isolated plasma membranes contain other enzymes such as myokinase, ATP pyrophosphohydrolase and 5′-nucleotidase, it seems

to be difficult to establish the definite sites of actions of these factors on each enzyme. Because of these reasons, and because the preparation of dopamine-β-bydroxylase coulde be highly purified, the properties and the site of action of the naturally occurring inhibitors of dopamine-β-hydroxylase (Izumi et al. 1975b, Oyama et al. 1975a, 1976) were investigated and the inhibition of dopamine-β-hydroxylase by various agents (Izumi et al. 1976b, c, d, f ; Oyama et al. 1975b) were further investigated.

Chapter 9. Materials and Methods

9-1. *Materials*

[^{3}H]ATP (uniformly labeled) was obtained from New England Nuclear Corp. [8-^{14}C]cyclic AMP was purchased from Schwarz Bioresearch. Crystalline adenosine 5'-triphosphate disodium salt (ATP), tris(hydroxymethyl)aminomethane and DL-norepinephrine were obtained from Sigma Chemical Co. Cyclic AMP was purchased from Daiichi Pure Chemical Co. Sodium fluride was obtained from Kanto Chemical Co. GTP was obtained from Kyowa Co. Ltd. Adenine and adenosine were obtained from Kohjin Co. Ltd. p-Chloromercuribenzoic acid sodium salt was purchased from Nakarai Chemical Ltd. All other chemicals were reagent grade preparations obtained from various commercial sources.

9-2. *preparation of Subcellular Fraction*

Sprague-Dawley male rats (CLEA Japan, Inc.), weighing 250-300 g, were used in the experiments. After animals were decapitated, brains were isolated and chilled in the ice-cold solution containing 0.32 M sucrose and 3.0 mM $MgSO_4$. Subsequently, the cerebral cortexs were isolated of Glowinski and Iversen (1966) and were homogenated in 9 volumes of the same isotonic cold solution using a glass homogenizer with a loose Teflon pestle. The homogenates were centrifuged at 4°C for 20 min at 1,000 × g. The supernatant fluid (1,000 × g supernatant) was pooled and centrifuged at 10,000 × g for 20 min at 4°C. The precipitate was diluted with the same sucrose medium and designated as the crude mitochondrial fraction (10,000 × g sediment). The supernatant was centrifuged at 105,000 × g for 60 min at 4°C. The precipitate was designated as the microsomal fraction (105,000 × g sediment) and the supernatant fraction as the supernatant (105,000 × g supernatant). The 105,000 × g supernatant was heated in a boiling water bath for 5 min and then, after 10-20 min at 0°C, centrifuged at 10,000 × g for 10 min at 4°C. This supernatant was used as the boiled supernatant.

9-3. *Assay of Cyclic AMP Phosphodiesterase*

Cyclic AMP phosphodiesterase was assayed by the method of Pöch (1971) using cyclic AMP as the substrate at the concentration of 3.6×10^{-4} M. The enzymic activity was measured as the rate of hydrolyzing cyclic AMP in a standard reaction medium (final volume 500 μl) containing 8-^{14}C-cyclic AMP (0.05 μCi), 3.0 mM Mg-acetate, 2.0 mM 5'-AMP, 100 mM Tris-HCl buffer (pH 7.4) and the enzymes. Incubations were run at 30°C for 15 min. The reaction was stopped by adding 200 μl of 0.17 M $ZnSO_4$ and 200 μl of 0.15 M $Ba(OH)_2$. After centrifugation (2,500 rpm, 10 min), 300

μl of the supernatant was transferred into a counting vial with 10 ml of scintillation medium (4 g of PPO, 100 mg of dimethyl POPOP, 100 ml of toluene, 400 ml of 99.5% ethanol and 100 ml of dioxane). The radioactivity was then determined by a liquid scintillation spectrometer (Packard Tri-Car Model 3380).

9-4. *Assay of ATPase*

Reaction mixtures (1.0 ml) contained 5.0 mM $MgCl_2$, 14 mM KCl, 140 mM NaCl, 50 mM Tris-HCl buffer (pH 7.4) and tissue suspension. The mixtures were shaken at 30°C for 5 min and the reaction was initiated by the addition of Tris-ATP (0.1 ml) to give a final concentration of 3.0 mM. Shaking was continued for a further 15 min, and 2.0 ml of 10% (W/V) trichloroacetic acid solution then added. Protein precipitate was spun down, and inorganic phosphate in samples was measured from the supernatant by the method of Fiske and Subbarow (1925).

9-5. *Electric Microscopic Observation*

Adult male rats (Sprague-Dawley, CLEA Japan, Inc.) weighing 300-350 g were used. After decapitation, the brain was quickly removed, and then the cerebral cortex was isolated by the method of Glovinski and Iversen (1966). The specimens were cut into small pieces. Synaptosomes were prepared by the method of Whittaker (1959) as shown in chapter 3.

Fixation was carried out for about two hours in 1% ice-cold osmium tetroxide. This fixatives were adjust to pH 7.4 with Millonig's phosphate buffer (Millonig 1962). They were then dehydrated in ethanol and transferred into propylene oxide, and embedded in Epon 812 (Luft 1961). Thin sections were stained with 1% uranyl acetate for ten minutes followed by Millonig's lead stain (Millonig 1961). A JEM-T7 electron microscope was used for observation.

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